Coa2 is an assembly factor for yeast cytochrome c oxidase biogenesis that facilitates the maturation of Cox1.

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Identification of Coa2 as an assembly factor for cytochrome *c* oxidase biogenesis

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ABSTRACT

The assembly of cytochrome *c* oxidase (CcO) in yeast mitochondria is dependent on a new assembly factor designated Coa2. Coa2 was identified from its ability to suppress the respiratory deficiency of *coa1*Δ and *shy1*Δ cells. Coa1 and Shy1 function at an early step in maturation of the Cox1 subunit of CcO. Coa2 functions downstream of Coa1 and the heme a_3 related function of Shy1. Cells lacking Coa2 exhibit a specific defect in CcO assembly and show a rapid degradation of newly synthesized Cox1. Rapid Cox1 proteolysis also occurs in *shy1*Δ cells; suggesting that in the absence of Coa2 or Shy1, Cox1 forms an unstable conformer. Overexpression of Cox5a and Cox6 or attenuation of the proteolytic activity of the m-AAA protease partially restores respiration in *coa2*Δ cells. The matrix localized Coa2 protein may aid in the assembly of an early Cox1 intermediate containing the nuclear subunits Cox5a and Cox6. We propose that Coa2 functions at a Cox1 assembly checkpoint where Cox1 assembly and degradation of stalled Cox1 intermediates are coupled.

INTRODUCTION

Cytochrome *c* oxidase (CcO) is the terminal enzyme of the energy transducing respiratory chain in mitochondria of eukaryotes. Eukaryotic CcO consists of 12-13 subunits, with three mitochondrial encoded subunits (Cox1-Cox3) forming the core enzyme embedded within the mitochondrial inner membrane (IM). The remaining nuclear encoded subunits pack on the periphery of the catalytic core (37). The core enzyme contains three copper atoms and two heme *a* cofactors (36).

The biogenesis of CcO, occurring within the IM, commences with the mitochondrial synthesis of the Cox1 subunit followed by the insertion of heme *a* and copper cofactors and the addition of the remaining subunits. The translation of Cox1 on mitoribosomes occurs in juxtaposition to the IM and is mediated in yeast by the IM-tethered translational activator Pet309 along with the IM-associated Mss51 (25, 26, 30, 32). The translational activators may recruit mitoribosomes and promote co-translational IM insertion together with the Oxa1 translocase (9, 18).

Insights have also been gleaned on the assembly process of CcO through characterization of stalled intermediate complexes in human cells of patients with mutations in assembly factors. One intermediate that accumulates in Leigh's syndrome patients with *SURF1* mutations consists of Cox1, CoxIV and CoxVa (34, 38, 41). This intermediate fails to accumulate in patient cells with mutations in Cox10 or Cox15, two enzymes that function in heme *a* biosynthesis. Thus, the prediction is that heme *a* insertion occurs prior to the addition of CoxIV and CoxVa (corresponding to yeast Cox5a/b and Cox6) (1, 2). Two heme *a* moieties and a copper ion are buried inside a barrel within Cox1 (36). These cofactors may be inserted through an interface obstructed by the association of the Cox2 subunit. Consistent with this postulate is the fact that Cox2 associates downstream of the Cox1, Cox5a, Cox6 complex (19).

Mss51, in conjunction with Cox14, has a post-translational function in yeast Cox1 maturation (8, 30). Mss51 and Cox14 form a complex that interacts with Cox1 and regulates Cox1 translation/elongation. In cells lacking the yeast *SURF1*, *shy1*Δ cells, Cox1 translation is attenuated (8). We recently reported that Coa1 functions in an early step of Cox1 assembly in yeast (31). Coa1 is part of the Mss51, Cox14, Cox1 complex and in addition binds to Shy1 (27, 31). Coa1 appears to coordinate the transition of newly synthesized Cox1 from the Mss51

complex to a later component involving Shy1 that likely functions in heme a_3 insertion. We isolated a series of extragenic suppressors of *coa1*Δ cells. High copy *MSS51*, *MDJ1* and *COX10* suppress the respiratory defect of *coa1*Δ cells. In addition, we observed that respiration in both *coa1*Δ and *shy1*Δ cells is enhanced when *MSS51* and *COX10* are co-expressed. The synergistic suppression is consistent with Mss51 and Cox10 affecting Cox1 translation and heme a_3 insertion suggesting that Coa1 links co-translational insertion and heme addition to Cox1.

A fourth extragenic suppressor of *coa1*Δ cells was found. High copy expression of the un-annotated yeast ORF, YPL189C-A, designated Coa2 for cytochrome oxidase assembly 2, is shown presently to effectively restore respiratory growth to *coa1*Δ and *shy1*Δ cells. We report that Coa2 functions in Cox1 maturation downstream from the Mss51 translational elongation step and at the heme a_3 insertion step involving Shy1.

MATERIALS AND METHODS

Yeast strains and vectors. Yeast strains used in this study are described in Table I. The deletions strains were created by disruption using homologous recombination of KanMX4 or *Candida albicans URA3*. Proper integration was checked by PCR of the locus and reversion of the phenotype induced by the deletion was insured by complementation with a WT copy of the gene on a plasmid. Cells were cultured in either rich medium or synthetic complete (SC) medium lacking the appropriate nutrients for plasmid selection. The carbon source used was either 2% glucose, 2% glycerol-2% lactate or 2% raffinose. *COA2* was cloned into YEp lac181 with 400 bp of its own promoter and terminator using BamHI and SalI. *COA2* ORF was cloned into pRS416 under the control of the *MET25* promoter and the *CYC1* terminator (28) using BamHI and SalI. *COX5a* and *COX6* were inserted in pRS426 and pRS423 under the control of their own promoter (600 and 430 bp respectively) and terminator (500 bp) by restriction with BamHI and XhoI. The vector containing Yta12 E614Q was a gift from Dr. Thomas Langer. Sequencing was used to confirm cloning products in all created vectors. Yeast strains were transformed using lithium acetate.

Mitochondria purification and assays. Intact mitochondria were isolated from yeast as described previously (13, 15). Total mitochondrial protein concentration was determined by Bradford assay (11). Cytochrome *c* oxidase activity was assessed by monitoring the oxidation of reduced cytochrome *c* and reaction rates were normalized to total mitochondrial protein (12). The oxygen consumption of the cells grown to stationary phase was determined on a 5300A Biological Oxygen Monitor (Yellow Springs Instrument Co.). The linear response was considered in calculating the rate of oxygen consumption. Heme analysis was conducted on purified mitochondria (1-2 mg protein) extracted with 0.5 mL of acetone containing 2.5% HCl as described previously (31). The pH of the extract was adjusted to 4 by addition of 1 μL formic acid and titration of a KOH solution. The sample was clarified by centrifugation at 13000 rpm for 5 min and 1 mL was injected onto a 3.9 x 300 mm C18 Bondclone column (Phenomex, USA).

Hydrogen peroxide sensitivity, respiratory growth assay and arginine auxotrophy. Sensitivity of the strains and transformants to hydrogen peroxide was assessed as previously described (22). To test respiratory competence, the strains were grown overnight in selective medium containing 2% raffinose - 0.2% glucose, serially diluted and spotted on 2% glucose containing medium (control) and 2% glycerol-2% lactate rich (YPLG) or synthetic complete medium (SCLG). The *cox1*Δ::*ARG8, COX1(1-512)*::*ARG8* strains and their derivatives were grown overnight in selective medium containing 2% glucose and 1x arginine (20 mg/mL Arg) or 0.2x Arg as indicated. The cultures were serially diluted and dropped onto synthetic complete medium 2% glucose containing no Arg or 1x Arg (control).

***In vivo* mitochondrial protein translation assay.** The cells were grown overnight in selective medium containing 2% raffinose and then re-inocculated in YP-2% raffinose to grow to an absorbance of 1 at 600 nm. The labeling and preparation of the samples for 12% SDS-PAGE or 15% SDS-PAGE (to visualize mp15) was as described in (7). The gel was dried and radiolabeled proteins were visualized by exposing autoradiographic films at -80°C.

***In vitro* import into isolated mitochondria.** *In vitro* import of Cox5a into isolated purified mitochondria was carried out as described (23) using Cox5a precursor protein labeled in a rabbit reticulocyte lysate (Promega, WI) with [^{35}S]-Met.

Blue native gel electrophoresis (BN-PAGE). BN-PAGE analysis was performed essentially as described (39) except that 1.5% digitonin was used. After incubation for 15 min on ice and centrifugation (20,000 g for 10 min at 2^{o}C), supernatants were mixed with sample buffer (5% Coomassie brilliant blue G250, 0.5 M 6-aminocaproic acid, pH 7.0) and loaded on 4-13% or 6-16% gradient polyacrylamide gel. Separated complexes were detected by immunoblotting on PVDF membrane or by autoradiography of dried gels.

Immunoblotting. Protein samples were separated on 15% acrylamide gels and transferred to nitrocellulose. Proteins were visualized using ECL reagents with horseradish peroxidase-conjugated secondary antibodies. Anti-Myc and HA antisera were purchased from Santa Cruz, anti-porin was from Molecular Probes and antisera to Cox1-Cox3 were from Mitosciences. Antiserum to Sod2 was provided by Dr. Val Culotta, and antisera to Cyb2 and Cyc1 were provided by Dr. Carla Koehler. Dr. Alex Tzagoloff provided antiserum to F1 ATP synthase and Dr. Peter Rehling provided antiserum to Cyt1.

RESULTS

Coa2 is a multicopy suppressor of *coa1*Δ and *shy1*Δ respiratory growth defect. In a search for high copy suppressors of the respiratory growth defect of *coa1*Δ cells, we recovered *MSS51*, *COX10* and *MDJ1* (31). A fourth suppressor, which we designated *COA2*, was also identified (Fig. 1A). Five plasmids recovered from the respiratory competent clones contained a DNA fragment encompassing YPL189c-a, an uncharacterized ORF that was identified by homology to an ORF in *Ashbya gossypii* (10, 20). Our identification of this gene as an extragenic suppressor and the respiratory deficiency of the deletion of YPL189c-a (10, 20) imply that YPL189c-a is a functional gene. The complementation of the respiratory growth defect of *coa1*Δ cells by overexpression of *COA2* is similar to that observed by overexpression of *MSS51* (Fig. 1A). Since Coa1 and Shy1 function at a related step in CcO

assembly (31), we tested whether *COA2* overexpression rescued the respiratory defect of *shy1*Δ cells. Coa2 overexpression was able to partially complement the respiratory growth defect of *shy1*Δ cells (Fig. 1B).

***coa2*Δ cells are respiratory deficient with a CcO specific defect.** The *COA2* ORF was deleted by homologous recombination with a KanMX4 cassette in BY4741 and W303 strain backgrounds. The *coa2*Δ strains showed normal growth on glucose medium but were unable to propagate on respiratory medium containing glycerol and lactate as carbon sources (Fig. 2A). Introduction of a centromeric plasmid expressing *COA2* under the control of the *MET25* promoter and *CYC1* terminator in the *coa2*Δ strains restored respiratory growth to WT level (Fig. 2B). Consistent with the growth defect observed on glycerol-lactate medium, oxygen consumption was strongly reduced in the *coa2*Δ strains compared to their isogenic WT (Fig. 2C). Overall, these results confirm that Coa2 is necessary for respiration in *S. cerevisiae* as was shown by Kastenmayer and coworkers (Kastenmayer et al., 2006).

Since Coa1 and Shy1 are part of the CcO assembly machinery, we assessed whether the absence of *COA2* led to a CcO defect. CcO activity measured on purified mitochondria was diminished to about 10% of WT in the *coa2*Δ strains (Fig. 3A). Heme *a*, a cofactor exclusive to CcO, was undetectable in mitochondrial extracts of *coa2*Δ strains (Fig. 3B). The bc_1 activity was reduced to 50-60% of WT and heme *b* levels were on average 58% of WT. Numerous CcO assembly mutants have reduced bc_1 activity. Immunoblotting showed an absence of Cox1-Cox3 in mitochondria purified from the *coa2*Δ strain, while cytochrome *c* and Qcr7, a subunit of complex III, were present at WT levels (Fig. 3C). Finally, CcO was undetectable by blue native PAGE (BN-PAGE) in the *coa2*Δ mitochondria (Fig. 3D). The absence of CcO caused complex III to be redistributed from the WT III_2/IV_2 and III_2/IV supercomplexes into the dimeric form (III_2) and the deletion of *COA2* had no effect on complex V (Fig. 3D). Overall, these data argue for a specific CcO defect in *coa2*Δ cells.

Coa2 is a mitochondrial matrix soluble protein partially associated with the IM. We chromosomally integrated three copies of the HA epitope at the 3'end of the *COA2* ORF. The tag did not affect the function of Coa2 as judged by the WT growth on respiratory medium of

the *COA2*-3HA strain (data not shown). Nycodenz gradient purified mitochondria isolated from the *COA2*-3HA strain showed a single band at ~17 kDa upon immunodetection with HA antiserum (Fig. 4A). This apparent mass is higher than the expected 10.8 kDa, although the basic isoelectric point of Coa2 (10.6) may account for its aberrant migration on SDS-PAGE. Coa2 was detected predominantly in the soluble fraction of sonicated mitochondria like the soluble proteins Cyc1 and Sod2 (Fig. 4B). When the concentration of sodium chloride was increased in the sonication buffer, Cyc1 lost its membrane association as expected, whereas the fraction of Coa2 recovered in the pellet increased (Fig. 4B). This salt effect suggests that Coa2 may have a hydrophobic interaction with a mitochondrial membrane or with one of its proteins. This candidate interaction may involve a highly hydrophobic domain between residues 13 and 33 that was assigned to be a transmembrane helix by several topology prediction programs. In order to determine the sub-compartmentalization of Coa2, we treated intact or hypotonically-lysed mitochondria with proteinase K. The inner membrane space protein Cyc1 was degraded in mitoplasts, whereas Coa2 was protected similarly to the matrix protein Sod2 (Fig. 4C). However, Coa2 was degraded by proteinase K upon solubilization of mitochondria with Triton X100 (data not shown). Coa2, like Sod2, was retained in the insoluble fraction of mitoplasts, whereas it was liberated in the soluble fraction upon sonication of mitochondria (Fig. 4D). On the contrary, Ccs1, an IMS soluble protein, was mostly present in the soluble fraction of mitoplasts. Taken together, these results show that Coa2 is a soluble protein in the mitochondrial matrix and that Coa2 may have an interaction with an IM anchored protein or the inner membrane itself.

The absence of *COA2* leads to low level of newly synthesized Cox1. *In vivo* labeling of mitochondrial translation products revealed that very limited amounts of Cox1 were detected in *coa2*Δ cells in both W303 and BY4741 backgrounds. Cox2, Cox3 and Var1 were translated at WT levels while Cob (Cyt *b*) translation was slightly increased (Fig. 5A and Supplementary Fig. 1). During the 60 min chase, the levels of Var1 and Cyt *b* remained stable, whereas Cox2 and Cox3 diminished markedly in *coa2*Δ cells, a phenomenon consistent with a CcO assembly defect. Low abundance of Cox1 during the pulse phase of *in vivo* mitochondrial translation experiments has been reported for most CcO mutants (8). This has been proposed to result from the accumulation of a Mss51:Cox14:Cox1 complex which titrates Mss51 out of

its Cox1 translational activator role (8). The synthesis of Cox1 can be restored in certain CcO mutants when Mss51 is overexpressed, when *COX14* is deleted (8) or when *COA1* is deleted (31). Deletion of *COX14* or *COA1* in *coa2*Δ cells restored WT levels of newly synthesized Cox1, whereas overexpression of Mss51 only partially returned Cox1 (Fig. 5B). However, none of these strains recovered respiratory growth (data not shown).

Recently, Barrientos and coworkers showed that attenuation of Mss51 levels or titration of Mss51 out of its Cox1 translational activator role leads to the synthesis of mp15, a truncated translation product of *COX1* mRNA in the W303 background (40). Mp15 was apparent in *cox5a*Δ cells, but its abundance was lower in *coa2*Δ cells (Fig. 5C). Consistent with reported data (40), mp15 synthesis was abrogated when *cox5a*Δ or *coa2*Δ cells were treated with chloramphenicol prior to labeling and the synthesis of some Cox1 was restored (Fig. 5C). The low abundance of mp15 in *coa2*Δ cells suggests that Mss51 is still able to fulfill its translation functions in the absence of *COA2*.

Cox1 is translated but rapidly degraded in *coa2*Δ cells. To evaluate the status of translation at the *COX1* locus in *coa2*Δ cells, we used Arg8 reporter strains constructed by Fox and coworkers (30). Arg8 is encoded by a nuclear gene and is imported to the mitochondrial matrix where it participates in the biosynthesis of arginine. These reporter strains constructed in an *arg8*Δ background, contain the *ARG8* gene in place of *COX1* ORF (*cox1*Δ::*ARG8*) or fused at the 3' end of the 512 codon intronless form of the *COX1* coding sequence (*COX1(1-512)*::*ARG8*) (30). The growth of these strains in medium lacking arginine is indicative of translation of *COX1(1-512)*::*ARG8* mRNA. Deletion of *COA2* in these two strains did not produce an arginine auxotrophy, implying that translation was occurring at the *COX1* locus (Fig. 6A). As expected, the *COX1(1-512)*::*ARG8* strain was unable to respire when *COA2* was deleted (Fig. 6A). To ascertain that the arginine prototrophy of the *cox1*Δ::*ARG8* and *COX1(1-512)*::*ARG8* strains lacking *COA2* was not sustained by only low amounts of Arg8, we performed *in vivo* mitochondrial translation assays in these strains. The level of mature Arg8 detected in the *cox1*Δ::*ARG8* strain was comparable whether *COA2* was present or not (Fig. 6B). In the *COX1(1-512)*::*ARG8* strain, a band corresponding to the unprocessed fusion was detected during the pulse phase of the reaction independently of the presence of *COA2* (Fig. 6C). The processing of this band into Cox1 and Arg8 during the chase was variable

between experiments. However, the level of total Arg8 (processed Arg8 and Cox1-Arg8 fusion) was comparable between the strains containing or not *COA2*, whereas the amount of processed Cox1 at the end of the chase was consistently greatly reduced in the absence of *COA2*. The experiments with the Arg8 reporter strains show that efficient translation occurs at the *COX1* locus even when the *COA2* gene is deleted.

We previously reported that Mss51 is distributed into 2 complexes when analyzed by BN-PAGE (31). The largest of this complex (complex A) has been postulated to contain Cox14, Coa1 and newly synthesized Cox1 based on the observations that this complex is disrupted upon deletion of *COX14* and is stabilized in *shy1*Δ cells (31). Complex A was resolved into two different complexes A1 and A2 when a gel with a 4-13% gradient was used (Fig. 6D). These two complexes were abrogated upon deletion of *PET309*, a *COX1* translational activator (Fig. 6D), supporting the assumption that newly synthesized Cox1 is required for the formation of complexes A1 and A2. These complexes were not significantly perturbed by the deletion of *COA2*, suggesting that newly synthesized Cox1 is present in this strain. Altogether, the data strongly suggest that the CcO deficiency in *coa2*Δ cells is not a result of a translation defect of Cox1.

The m-AAA protease composed of Yta10-Yta12 is known to degrade non-assembled mitochondrially encoded proteins within the IM (4). Additionally, the m-AAA protease is essential for mitoribosome assembly through processing of MrpL32 (35). To evaluate whether the m-AAA protease was responsible for the low level of newly synthesized Cox1 in *coa2*Δ cells, we used a mutant m-AAA containing a E614Q substitution in Yta12 that attenuates its proteolytic activity yet maintains its processing function (3, 35). Respiratory growth was partially restored in *coa2*Δ cells when *YTA12* was deleted and replaced with the Yta12 E614Q allele (Fig. 6E). Therefore, rapid degradation of Cox1 contributes to the CcO deficiency in *coa2*Δ cells.

Coa2 acts downstream of Shy1, Coa1 and Cox14 in CcO assembly pathway. Deletion of certain CcO assembly factors, such as Cox11 and Sco1, render yeast cell sensitive to hydrogen peroxide (6), a phenotype generated by a pro-oxidant heme a_3-containing Cox1 assembly intermediate (22). We found that *coa2*Δ cells are also sensitive to hydrogen peroxide in a Cox1-dependent way, as the *cox1*Δ::*ARG8* strain did not exhibit sensitivity in

the absence of *COA2* (Fig. 7A). Thus, despite low levels of Cox1 observed in the [^{35}S] translation assay in *coa2*Δ cells, sufficient Cox1 was present to cause the peroxide sensitivity. This highlights the sensitivity of the hydrogen peroxide assay. We showed previously that peroxide sensitivity was dependent on heme a_3 insertion into Cox1 (22). We took advantage of this phenotype to define the step in CcO assembly at which Coa2 is involved. Deletion of *COA1*, *SHY1* or *COX14* in *coa2*Δ cells abrogated the peroxide sensitivity (Fig. 7B), despite the elevated levels of newly synthesized Cox1 detected in *coa2*Δ,*coa1*Δ and *coa2*Δ,*cox14*Δ cells (Fig. 5B). Deletion of *COA1* or *COX14* must preclude heme a_3 insertion as is the case with *SHY1* disruption. These data together with the fact that overexpression of Coa2 can suppress *coa1*Δ and *shy1*Δ cells strongly support a role for Coa2 downstream of Coa1, Cox14 and Shy1 in the assembly pathway of CcO.

The respiratory defect of *coa2*Δ cells is suppressed by overexpression of Cox5a and Cox6. Cox1 occupies a central position in the mature CcO structure (37). We reasoned that the rapid degradation of Cox1 in *coa2*Δ cells likely occurs at an early stage in CcO assembly. In humans, CoxIV and CoxVa (yeast Cox5a and Cox6) associate with Cox1 as the first subassembly intermediate (29, 38) and these subunits are in close contact with Cox1 in the bovine CcO (Fig. 8A). We addressed whether Cox5a and Cox6 have a significant effect on Cox1 stability at early stages of CcO assembly. We performed *in vivo* mitochondrial translation experiments in strains carrying deletion of different CcO subunits, early assembled subunits like Cox5a and Cox6 and subunits assembled at a latter stage like Cox7 and Cox12. Only minimal levels of Cox1 were detected in *cox5a*Δ and *cox6*Δ cells, whereas *cox7*Δ and *cox12*Δ cells showed WT levels of Cox1 during the pulse of the reaction (Fig. 8B). This result prompted us to overexpress Cox5a and Cox6 in *coa2*Δ cells. The two genes, when overexpressed individually, showed suppression of *coa2*Δ respiratory defect at 37°C (Fig. 8C). However, Cox5a and Cox6 had to be overexpressed together to produce significant growth of *coa2*Δ cells at 30°C (Fig. 8C). Coa2 is probably not required for Cox5a stability *per se*, since the same residual level of Cox5a was detected in *coa2*Δ or in *cox19*Δ cells (Fig. 8D). Cox19 is another CcO assembly factor. Coa2 may therefore aid in complex formation between Cox5a and newly synthesized Cox1.

Genomically tagged Coa2 migrated on BN-PAGE as a single complex whose mass was approximately 100 kDa (Fig. 8E). This is in contrast to the predicted mass of 27 kDa for Coa2-13Myc. The observed Coa2 complex is smaller that any of the Cox5a containing complexes described recently (27) suggesting that Coa2 may not form a stable complex with Cox5a. Immunoprecipitation experiments between Coa2 and Cox5a failed to show an interaction between the two proteins (data not shown). In addition, import of radiolabeled Cox5a into purified mitochondria from WT cells and cells containing Coa2-13Myc showed the same mass complexes (Supplemental Fig. 2). A mass shift was shown to occur in the import of radiolabeled Cox5a in mitochondria containing an epitope-tagged Shy1 relative to mitochondria with an untagged Shy1 (27). Due to the low Met and Cys content of Coa2, we engineered a C-terminal extension with 6 methionine residues. However, attempts to radiolabel Coa2 *in vitro* with ^{35}S were unsuccessful, precluding experiments to assess the association of Coa2 in transient complexes upon *in vitro* import. The 100 kDa Coa2 complex does not appear to contain Cox1, since this complex on BN-PAGE was apparent in *pet309*Δ cells in which Cox1 is not translated (data not shown). We failed to detect an interaction between Coa2 and newly synthesized Cox1 when Coa2 was immunoprecipitated from radiolabeled mitochondria (data not shown). However, not all IM complexes are stably maintained under BN-PAGE conditions (14).

***COA2* and *MSS51* have non-redundant complementary functions.** As in *coa2*Δ cells, Cox1 synthesis is also unusually low in *cox5a*Δ cells. In order to test whether the low level of Cox1 detected in *cox5a*Δ (Fig. 8A) was a result of lack of translation or rapid degradation, we disrupted *COX5a* in the *COX1(1-512)::ARG8* strain. This strain showed a partial arginine auxotrophy which was reversed by overexpression of *MSS51* or *COA2* (Fig. 9A). Consistent with the Cox1 translation block model (8), overexpression of *MSS51* in a *cox5a*Δ strain partially returned the level of newly synthesized Cox1 while *COA2* overexpression did not (Fig. 9B). These results suggest that *MSS51* probably increases translation of the Cox1-Arg8 fusion to rescue the arginine auxotrophy of the *COX1(1-512)::ARG8 cox5a*Δ strain, whereas *COA2* suppression may arise in a post-translational step through stabilization of the translated Cox1-Arg8. The low intensity of this band by *in vivo* mitochondrial translation assay in the *COX1(1-512)::ARG8 cox5a*Δ strain prevented us from directly testing this hypothesis.

We subsequently assayed the peroxide sensitivity of the *cox5a*Δ strain to assess heme a_3:Cox1 levels. Cells lacking Cox5a were not sensitive unless *MSS51* or *COA2* were overexpressed (Fig. 9C). The coexpression of the two proteins further increased the peroxide sensitivity (Fig. 9C), arguing for non-redundant functions of *MSS51* and *COA2*. High levels of Mss51 and Coa2 appear to enhance Cox1 levels, via translational and post-translational effects respectively.

A synergistic effect of *MSS51* and *COA2* was also found in the suppression of the respiratory defect of *coa1*Δ, a strain that has impaired CcO assembly due to a post-translational defect (Fig. 10A). Similarly, co-overexpression of *MSS51* and *COA2* improved the growth of a *shy1*Δ strain (Fig. 10B). Taken together, these results suggest that Coa2 is involved in Cox1 maturation at a post-translational step after the heme a_3 insertion mediated by Shy1. The respiratory defect of a *shy1*Δ strain was recently shown to be partially suppressed by overexpression of Cox5a and Cox6 (16). We did not observed any synergism in the suppression of a *shy1*Δ strain when these 2 subunits were overexpressed in combination with Coa2 (data not shown) suggesting that Coa2, Cox5a and Cox6 function at a related step in CcO assembly.

DISCUSSION

Coa2 is a 68 residue mitochondrial protein shown to be required for respiration and having a specific role in CcO assembly. The most striking aspect of the CcO deficiency of *coa2*Δ cells is a rapid degradation of newly synthesized Cox1. Only limited quantity of newly synthesized Cox1 is observed with *in vivo* mitochondrial translation and this limitation results from rapid degradation rather than impaired synthesis. Two lines of evidence support this conclusion. First, deletion of *COA2* in a *COX1(1-512)*::*ARG8* fusion strain resulted in normal growth on medium lacking arginine, whereas deletion of *COX5a* in the same background induced an Arg auxotrophy partially due to restricted translation. Second, respiration was partially recovered in *coa2*Δ cells by diminishing the activity of the m-AAA protease, a complex which has been shown to degrade misfolded inner membrane proteins (5).

COX1 translation is impaired in many CcO assembly mutants due to the titration of Mss51 into a post-translational complex, preventing its function as a translational activator of *COX1* (8). This *COX1* translation block is unlikely or at least limited in *coa2*Δ cells, since Arg8 is translated essentially normally in *COX1(1-512)*::*ARG8 coa2*Δ cells. The attenuation of Mss51 as a translational activator is also known to result in synthesis of a truncated Cox1 translation product called mp15 (40). Whereas mp15 synthesis occurs in *cox5a*Δ cells, only limited synthesis of mp15 occurs in *coa2*Δ cells.

A translation block of Cox1 likely exists in the absence of *COX5a* as shown by the rescue of the Arg auxotrophy of *COX1(1-512)*::*ARG8 cox5a*Δ cells by *MSS51* overexpression and by the recovery of some level of newly synthesized Cox1 in a *cox5a*Δ strain overexpressing *MSS51*. The translation block occurring in most CcO mutants can be obviated in cells lacking Coa1 or Cox14. The deletion of *COX14* leads to a marked enhancement in Cox1 synthesis, but the newly synthesized Cox1 does not progress to assembled CcO (8). Deletion of either *COA1* or *COX14* in *coa2*Δ cells leads to a pronounced increase in newly synthesized Cox1 in an *in vivo* mitochondrial translation assay. The accumulated Cox1 must be in an environment where it is protected from the protease responsible of its rapid degradation in *coa2*Δ cells. Cox14 and Coa1 are essential to coordinate Mss51's post-translational function leading to cofactor insertion in Cox1 (31). Therefore, disruption of *COX14* or *COA1* likely prevents newly synthesized Cox1 from joining the assembly chain. Indeed the reversal of the peroxide sensitivity of *coa2*Δ cells by deletion of *COX14* or *COA1*, even though it increases greatly the amount of newly synthesized Cox1 visible in the translation assay, suggests that this Cox1 is devoid of heme a_3. Shy1 has been shown to be important for heme a_3 insertion in *Rhodobacter* (33), and the fact that deletion of *SHY1* in *coa2*Δ cells also reverses the peroxide sensitivity supports this point.

At what step in CcO assembly is Coa2 important? Three lines of evidence suggest that Coa2 functions downstream of Coa1 and the heme a_3 related function of Shy1. First, the Coa1:Mss51:Cox14:Cox1 complex is unaffected in *coa2*Δ cells. Second, Cox1 can acquire heme a_3 in the absence of *COA2*, a step that is abrogated by the deletion of *COA1* and *SHY1* as shown by the reversal of the peroxide sensitivity of *coa2*Δ cells. Third, overexpression of *COA2* suppresses the respiratory phenotype of *coa1*Δ and *shy1*Δ cells. These results strongly point to Coa2 acting downstream of these two assembly factors. Although Shy1 appears to be

important in the heme a_3 insertion step, Shy1 stays associated with CcO until the supercomplex stage (27). It remains unclear whether Shy1 has any function downstream of the proposed heme a_3 assembly step.

Proteolytic degradation of Cox1 may be a protective response to the assembly of the pro-oxidant heme a_3:Cox1 intermediate. The enhanced activity in *coa2*Δ cells may suggest that Coa2 functions at a Cox1 assembly checkpoint that couples the progression of Cox1 assembly and degradation of stalled heme a_3:Cox1 intermediates. In the absence of heme a_3 insertion, Cox1 assembly intermediates, e.g. in *cox14*Δ cells, are not deleterious and perhaps not a priority for the cell to remove proteolytically.

We show that *COX5a* and *COX6* partially suppress the respiratory defect of *coa2*Δ, arguing for Coa2 functioning upstream or proximate to these two subunits joining Cox1. *COX5a* and *COX6* were recently shown to partially suppress the respiratory defect of *shy1*Δ cells (16). In this case, the authors suggested that Cox5a and Cox6 protect newly synthesized Cox1 from proteolytic degradation. Therefore, it is possible that at least part of the decrease in the amount of newly synthesized Cox1 observed in *shy1*Δ actually results from rapid degradation of Cox1 and not entirely from a translation block. This argument is supported by the observation that a *COX1(1-512)*::*ARG8 shy1*Δ strain has no growth defect on medium lacking Arg (30) and that normal level of Arg8 are present in the *COX1(1-512)*::*ARG8* strain in the absence of *SHY1* (30). Therefore, the same rapid Cox1 proteolysis may occur in *shy1*Δ as well as in *coa2*Δ cells suggesting that in the absence of these factors Cox1 forms an unstable conformer. Given the close packing of Cox5a against Cox1, Cox5a may have a stabilizing effect on newly synthesized Cox1.

An early step in Cox1 maturation is formation of the Cox1:Cox5a:Cox6 subassembly complex (34, 38). Cox5a packs against transmembrane helices 11 and 12 in Cox1 and together with Cox6 packs onto the matrix face of the Cox1 helical barrel stabilizing loops connecting the 12 membrane helices. This complex accumulates in mammalian cells with mutations in SURF1 (Shy1) (34, 38). This assembly intermediate likely contains heme *a*, since mutant Cox10 or Cox15 fibroblast lines fail to form this intermediate (1, 2). Heme *a* is axially coordinated by histidyl residues from Cox1 helices 2 and 10 and its farnesyl tail packs between helices 1, 11 and 12 (36). Thus, heme *a* insertion may assist in organizing the Cox1 helices creating the Cox5a interface.

It is conceivable that heme *a* produced by Cox10/Cox15 and inserted into newly synthesized Cox1 creates a Cox1 conformer competent for Cox5a,Cox6 binding. The heme a_3:Cu_B site may be formed in a second step mediated by Shy1 and Cox11. These two steps may occur concurrently rather than sequentially. In support of concurrent steps, a 350 kDa Cox1 assembly intermediate contains both Shy1 and Cox5a, although distinct 250 kDa complexes exist with either Shy1 or Cox5a (27). Coa2 may have a chaperone activity in promoting Cox5a/Cox6 association with Cox1, but appears independent of the Shy1 step. The inability to efficiently form the Cox1:Cox5a:Cox6 intermediate in *coa2*Δ cells could expose Cox1 to rapid proteolysis. In *shy1*Δ cells the unstable Cox1 intermediate may lack heme a_3. Coa2 likely suppress *shy1*Δ respiratory defect via a post-translational effect on Cox1. We did not detect any enhancement of the suppression of *shy1*Δ by Coa2 when Cox5a and Cox6 were also overexpressed. Therefore, it is probable that Coa2 suppresses the respiratory defect of *shy1*Δ cells by a similar mechanism as Cox5a and Cox6 which was proposed to be due to increasing the stability of newly synthesized Cox1 (16). Such a role of Coa2 would also explain the H_2O_2 sensitivity of a *cox5a*Δ strain overexpressing Coa2. Even though we failed to detect any stable interaction between Coa2 and Cox1 or Cox5a, a transient interaction may occur. Coa2 showed a salt-dependent association with the membrane fraction consistent with a hydrophobic interaction that could involve one of these protein. Future studies will focus on the components in the Coa2 complex.

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Figure Legends

Fig. 1. Overexpression of *COA2* can suppress the respiratory growth defect of *coa1Δ* and *shy1Δ* cells. A) *coa1*Δ cells (BY4743 background) transformed with episomal vectors expressing *MSS51, MDJ1,COX10* or *COA2* were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate (SCLG) or 2% glucose. The plates were incubated at 30°C for 2 days (glucose) or 7 days (SCLG). **B)** *shy1*Δ cells (W303 background) transformed with an episomal vector expressing *COA2* or an empty vector were grown as in A) and spotted on YP 2% glycerol-2% lactate (YPLG) or 2% glucose. The plates were incubated at 30°C for 2 days (glucose) or 4 days (YPLG).

Fig. 2. *coa2Δ* cells are respiratory deficient. A) *coa2*Δ cells and the isogenic WT (BY4741 or W303) were grown in liquid YPD then serially diluted and spotted on YP 2% glycerol-2% lactate (YPLG) or 2% glucose plates (YPD). The plates were incubated at 30°C for 2 days (YPD) or 4 days (YPLG). **B)** *coa2*Δ, the isogenic WT and *coa2*Δ cells transformed with a centromeric vector expressing *COA2* ORF under the control of the *MET25* promoter and the *CYC1* terminator were grown in SC-2% glucose, serially diluted and spotted on YPD or SC-2% glycerol-2% lactate (SCLG). The plates were incubated as in A). **C)** *coa2*Δ cells and the

WT were grown in liquid YPD at 30°C and oxygen consumption (% O_2/sec/OD_{600}) was measured. The data represent the average of 4 independent experiments.

Fig. 3. Deletion of *COA2* results in a CcO specific defect. **A)** *coa2*Δ cells and the isogenic WT were grown in YP-2% raffinose, mitochondria were purified and assayed for CcO activity (ΔOD_{550}/min/10 µg protein). The data represent the average of 3 independent experiments. **B)** Heme was extracted from mitochondria (2 mg protein) purified from *coa2*Δ or W303 cells and separated by reverse phase HPLC. The peaks corresponding to heme *b* (B) and heme *a* (A) and protoporphyrin (P) are shown. **C)** Immunoblot of mitochondria (30 µg protein) from *coa2*Δ cells or from wild-type W303. **D)** Mitochondria (200 µg protein) isolated from either wild-type (WT) or *coa2*Δ strains were solubilized in buffer containing 1.5% digitonin. Lysates were loaded onto a continuous 4-13% gradient gel and protein complexes were separated by BN-PAGE. The distribution of respiratory complexes was analyzed by immunoblotting with antisera against Cyt1, Cox2 and F_1-subunit.

Fig.4. Coa2 is a soluble matrix protein partially associated with the inner-membrane. A) Immunoblot of mitochondria and post-mitochondrial fraction (PMF) purified from the *COA2*-3HA strain. Pgk1 is a cytosolic marker and Por1 is a mitochondrial outer-membrane (OM) protein. **B)** Purified mitochondria from the *COA2*-3HA strain were sonicated in Hepes 20 mM pH 7.4 and the indicated concentration of NaCl. The soluble and insoluble fractions were separated by centrifugation at 100,000 g for 1 h and analyzed by immunoblot. Cyt1 is an integral inner-membrane (IM) protein, Cyc1 is associated with the IM and Sod2 is a matrix soluble protein. **C)** Purified mitochondria from the *COA2*-3HA strain were incubated in Hepes 20 mM pH 7.4 with 1.2 M Sorbitol (intact) or without it (hypotonic) for 30 min on ice, digested on ice for 40 min with the indicated concentration of proteinase K (PK) (µg/mL) and analyzed by immunoblotting. **D)** Purified mitochondria from the *COA2*-3HA strain were sonicated or incubated in Hepes 20 mM pH 7.4 with 1.2 M Sorbitol (intact) or without it (hypotonic) for 30 min on ice. The soluble (S) and insoluble fractions (P) were separated by centrifugation at 15,000 g for 20 min and analyzed by immunoblot. Ccs1 is a soluble IMS protein.

Fig. 5. The absence of *COA2* leads to low level of newly synthesized Cox1. A) *In vivo* labeling of mitochondrial translation products. *coa2*Δ and WT cells were pulsed for 5 and 15 min with [^{35}S]-methionine. After 15 min, 20 mM of cold methionine (Met) was added and the reaction was chased for 20 and 60 min at 30°C. The samples were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film. **B)** Cells were labeled for 8 and 20 min with [^{35}S]-Met at 30°C. The samples were analyzed as in A). **C)** W303 *coa2*Δ or *cox5a*Δ cells were treated (+) or not (-) for 3 h with 2 mg/mL chloramphenicol (CAP) and then pulsed for 20 min with [^{35}S]-Met. The samples were run on 15% SDS-PAGE, the gel was dried and exposed to autoradiographic film.

Fig. 6. Cox1 is translated in the absence of *COA2*. A) *COA2* was deleted in *arg8*Δ strains in which *ARG8* replaces the *COX1* codons (*cox1*Δ::*ARG8*) or *ARG8* is fused to 512 codons of *COX1* (*COX1*(1-512)::*ARG8*). The strains were grown in SC-2% glucose containing 0.2x Arg (4 mg/L), serially diluted and spotted on YPD, SC-2% glucose with or without 1x Arg and YPLG. The plates were incubated at 30°C for 2 days (glucose plates) or 4 days (YPLG). The *arg4*Δ strain is shown as a control. **B)** *In vivo* labeling of mitochondrial translation products of the *cox1Δ*::*ARG8* strain with or without the *COA2* gene. The cells were treated as in Fig.5A. The additional band is the mature Arg8. **C)** The *COX1(1-512)::ARG8* strain with or without the *COA2* gene was radiolabeled as in Fig.5A. The bands corresponding to the Cox1::Arg8 fusion and to the mature Arg8 are marked. **D)** Mitochondria (250 µg protein) isolated from either wild-type (WT), *coa2*Δ or *pet309*Δ strains containing a genomically tagged 13Myc *MSS51* (*MSS51::*Myc) were solubilized in buffer containing 1.5% digitonin. Lysates were loaded onto a continuous 4-13% gradient gel and protein complexes were separated by BN-PAGE. The distribution of Mss51 complexes was analyzed by immunoblotting with anti-Myc antibody. **E)** *coa2*Δ *yta12*Δ cells transformed with a centromeric vector expressing Yta12 E614Q were grown in SC-2% raffinose, serially diluted and spotted on YP 2% glycerol-2% lactate (YPLG) or 2% glucose (YPD). The plates were incubated at 30°C for 2 days (YPD) or 6 days (YPLG).

Fig. 7. Coa2 acts downstream of Coa1 and Shy1 in the assembly pathway of CcO. A) Cells were grown to mid-exponential phase and incubated with (+) or without (–) the

indicated concentration of H_2O_2 for 2 h at 30 °C. Serial dilutions were spotted onto YPD plates and incubated for 36–48 h at 30 °C. **B)** Cells (W303 background) were treated like in A).

Fig. 8. Overexpression of Cox5a can partially rescue the respiratory deficiency of *coa2Δ* cells. A) View of Cox1, Cox5a and Cox6 based on the structure of bovine CcO (37). Cox1 is shown in light gray, Cox5a and Cox6 are shown in black and dark gray, respectively. **B)** *In vivo* labeling of mitochondrial translation products in cells lacking CcO subunits. Cells (BY4743 background) were labeled for 8 and 20 min with [^{35}S]-Met at 30°C. The samples were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film. **C)** W303 *coa2*Δ cells transformed with episomal vectors expressing *COX5a* or *COX6* were grown in SC-2% raffinose, serially diluted and spotted on YP 2% glycerol-2% lactate (YPLG) or 2% glucose (YPD). The plates were incubated at 30°C and 37°C for 2 days (YPD) or 7 days (YPLG). **D)** Immunoblot of TCA extracts from W303, *coa2*Δ and *cox19*Δ cells containing a centromeric vector expressing a 3HA C-terminal tagged version of *COX5a* under the control of its own promoter. Porin (Por1) is shown as a loading control. **E)** Mitochondria isolated from either wild-type (WT) or a strain containing a genomically tagged 13Myc *COA2* (*COA2::*Myc) were solubilized in buffer containing 1.5% digitonin. Lysates were separated by BN-PAGE (6-16% gradient gel) and analyzed as in Fig.6D. Monomeric (V_1) and dimeric (V_2) forms of respiratory chain complex V served as a control and were visualized using antisera against F_1-subunit.

Fig.9. Coa2 and Mss51 overexpression in *cox5aΔ* cells results in increased levels of Cox1. A) The *COX1(1-512)::ARG8* strain lacking *COX5a* and with or without episomal vectors expressing *COA2* or *MSS51* were grown in SC-2% glucose containing 1x Arg (20 mg/L), serially diluted and spotted on SC-2% glucose containing 1x Arg (+Arg) or not (-Arg). The plates were incubated at 30°C for 2 days. **B)** W303 *cox5a*Δ cells containing the same plasmids as in A) were labeled for 8 and 20 min with [^{35}S]-Met at 30°C. The samples were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film. **C)** The same cells as in B) were grown to midexponential phase and incubated with (+) or without (–) the indicated

concentration of H_2O_2 for 2 h at 30 °C. Serial dilutions were spotted onto YPD plates and incubated for 36–48 h at 30 °C.

Fig.10. Coa2 and Mss51 have a synergistic effect in enhancing respiration of *coa1Δ* and *shy1Δ* cells. A) BY4743 *coa1*Δ cells transformed with episomal vectors expressing *MSS51, COA2* or both were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate (SCLG) or 2% glucose. The plates were incubated at 30°C for 2 days (glucose) or 6 days (SCLG). **B)** BY4743 *shy1*Δ cells transformed with the same vectors as in A) were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate (SCLG) or 2% glucose or YP 2% glycerol-2% lactate (YPLG). The plates were incubated at 30°C for 2 days (glucose) or 6 days.

Table I

Yeast strains used in this study

The strains with the 3 HA or 13Myc tags were generated in the DY5113 background by PCR-based gene modification using the template pFA6a-3HA-*TRP1*, pFA6a-13MYC-*TRP1*, pFA6a-3HA-*HIS3*MX6 or pFA6a-13MYC-*HIS3*MX6 (24).

Strain	Genotype	Reference/Source
BY4741	MATa, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Invitrogen Corp.
BY4741 Δ*coa2*	MATa, *his3*Δ1, *leu2*Δ0, *met15*Δ0 *ura3*Δ0, Δ*coa2:: kan*MX4	This work
W303	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1*	
W303 Δ*coa2*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*coa2:: kan*MX4	This work
W303 Δ*coa2* Δ*cox14*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*coa2:: kan*MX4, Δ*cox14:: Ca URA3*	This work
W303 Δ*coa2*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*coa2:: Ca URA3*	This work
W303 Δ*coa2* Δ*yta12*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*coa2:: Ca URA3,* Δ*yta12:: kan*MX4	This work
W303 Δ*coa2* Δ*coa1*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*coa2:: Ca URA3,* Δ*coa1:: kan*MX4	This work
W303 Δ*shy1*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*shy1::URA3*	(7)
W303 Δ*shy1* Δ*coa2*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*shy1::URA3,* Δ*coa2:: kan*MX4	This work
W303 Δ*cox5a*	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1,* Δ*cox5a:: HIS3*	(17)
*cox1*Δ*::ARG8m*	MATa, *lys2*, *leu2-3,112*, *arg8::hisG*, *ura3-52* [*cox1*Δ::*ARG8*m]	(30)
*cox1*Δ*::ARG8m* Δ*coa2*	MATa, *lys2, leu2-3,112*, *arg8::hisG*, *ura3-52* [*cox1*Δ::*ARG8*m], Δ*coa2:: kan*MX4	This work
COX1(1- 512)::ARG8	MATa, *lys2*, *leu2-3,112*, *arg8::hisG*, *ura3-52*, [*COX1*(1- 512)::*ARG8*m]	(30)
COX1(1- 512)::ARG8 Δ*coa2*	MATa, *lys2*, *leu2-3,112*, *arg8::hisG*, *ura3-52*, [*COX1*(1- 512)::*ARG8*m], Δ*coa2:: kan*MX4	This work
COX1(1- 512)::ARG8 Δ*cox5a*	MATa, *lys2*, *leu2-3,112*, *arg8::hisG*, *ura3-52*, [*COX1*(1- 512)::*ARG8*m], Δ*cox5a:: kan*MX4	This work
DY5113	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1*	(21)
COA2-3HA	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1, COA2*-3HA*::TRP1*	This work
COA2-13Myc	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1, COA2*-13Myc*::TRP1*	This work
COA2-13Myc Δ*pet309*	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1, COA2*-13Myc*::TRP1,* Δ*pet309:: CaURA3*	This work
MSS51-13Myc *COA1*-3HA	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1, COA1*-3HA*::TRP1, MSS51*-13Myc*::HIS3*MX6	(31)
MSS51-13Myc *COA1*-3HA Δ*coa2*	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1, COA1*-3HA*::TRP1, MSS51*-13Myc*::HIS3*MX6, Δ*coa2:: kan*MX4	This work
MSS51-13Myc *COA1*-3HA Δ*pet309*	MATa *ade2-1 his3-1,15 leu2-3,112 trp1*Δ*, ura3-1, COA1*-3HA*::TRP1, MSS51*-13Myc*::HIS3*MX6, Δ*pet309:: CaURA3*	This work
BY4743 Δ*shy1*	MATa/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2*/*lys2*Δ0 *MET15*/*met15*Δ0 *ura3*Δ0/*ura3*Δ0 Δ*shy1*:: *kan*MX4	Invitrogen Corp.
BY4743 Δ*coa1*	MATa/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2*/*lys2*Δ0 *MET15*/*met15*Δ0 *ura3*Δ0/*ura3*Δ0 Δ*coa1*:: *kan*MX4	Invitrogen Corp.
BY4743	MATa/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2*/*lys2*Δ0 *MET15*/*met15*Δ0 *ura3*Δ0/*ura3*Δ0	Invitrogen Corp.
BY4743 Δ*cox5a*	MATa/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2*/*lys2*Δ0 *MET15*/*met15*Δ0 *ura3*Δ0/*ura3*Δ0, Δ*cox5a:: kan*MX4	Invitrogen Corp.
BY4743 Δ*cox6*	MATa/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2*/*lys2*Δ0 *MET15*/*met15*Δ0 *ura3*Δ0/*ura3*Δ0, Δ*cox6:: kan*MX4	Invitrogen Corp.
BY4743 Δ*cox7*	MATa/α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *LYS2*/*lys2*Δ0 *MET15*/*met15*Δ0	Invitrogen Corp.

	*ura3*Δ0/*ura3*Δ0, Δ*cox7:: kan*MX4	

1

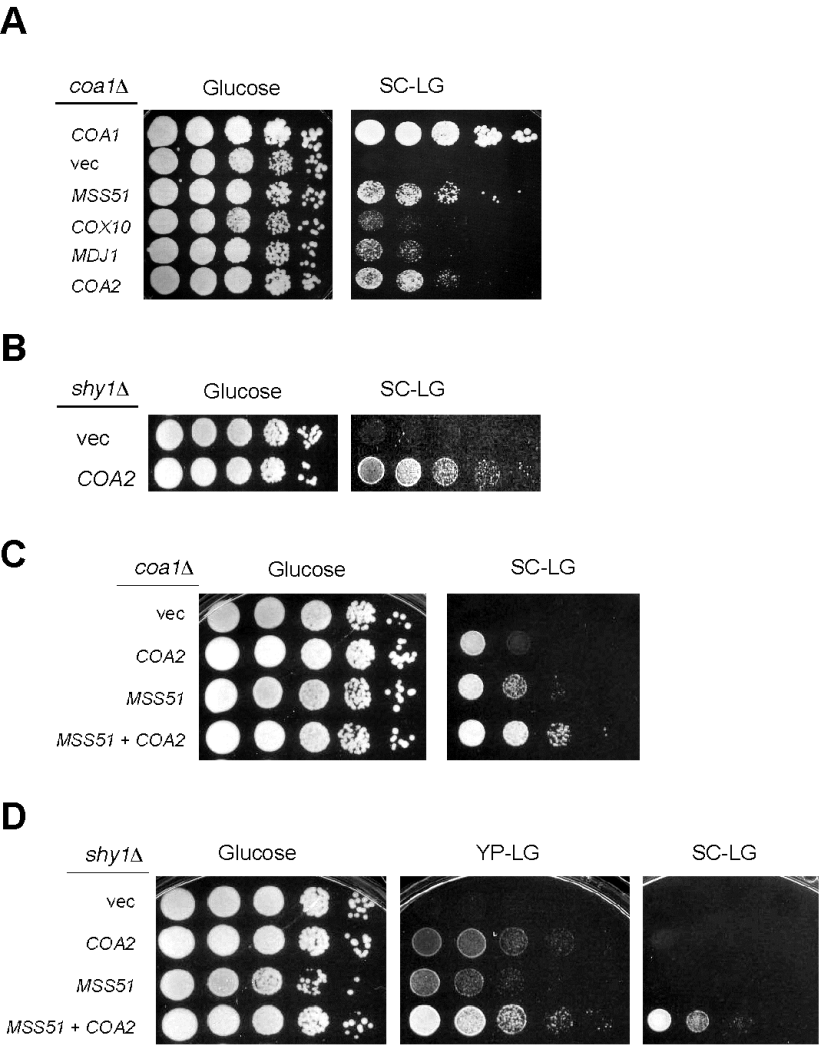
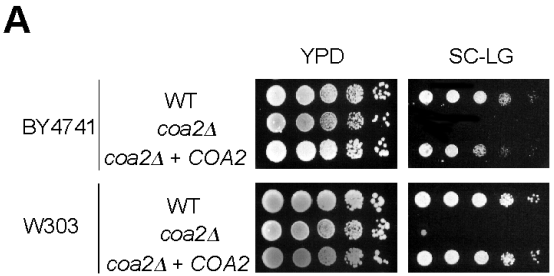

A
coa1Δ
Glucose
SC-LG
COA1
vec
MSS51
COX10
MDJ1
COA2
B
shy1Δ
Glucose
SC-LG
vec
COA2
C
coa1Δ
Glucose
SC-LG
vec
COA2
MSS51
MSS51 + COA2
D
shy1Δ
Glucose
YP-LG
SC-LG
vec
COA2
MSS51
MSS51 + COA2

Figure 1


A
YPD
SC-LG
BY4741
WT
coa2Δ
coa2Δ + COA2
W303
WT
coa2Δ
coa2Δ + COA2

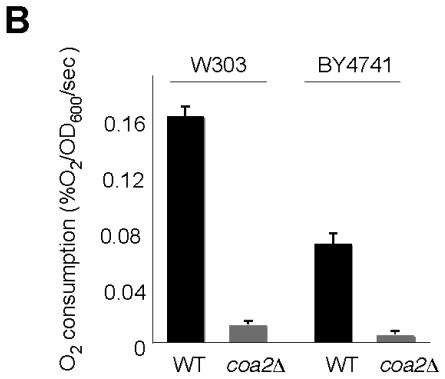

B
W303
BY4741
O2 consumption (%O2/OD600/sec)
0.16
0.12
0.08
0.04
0
WT
coa2Δ
WT
coa2Δ

Figure 2

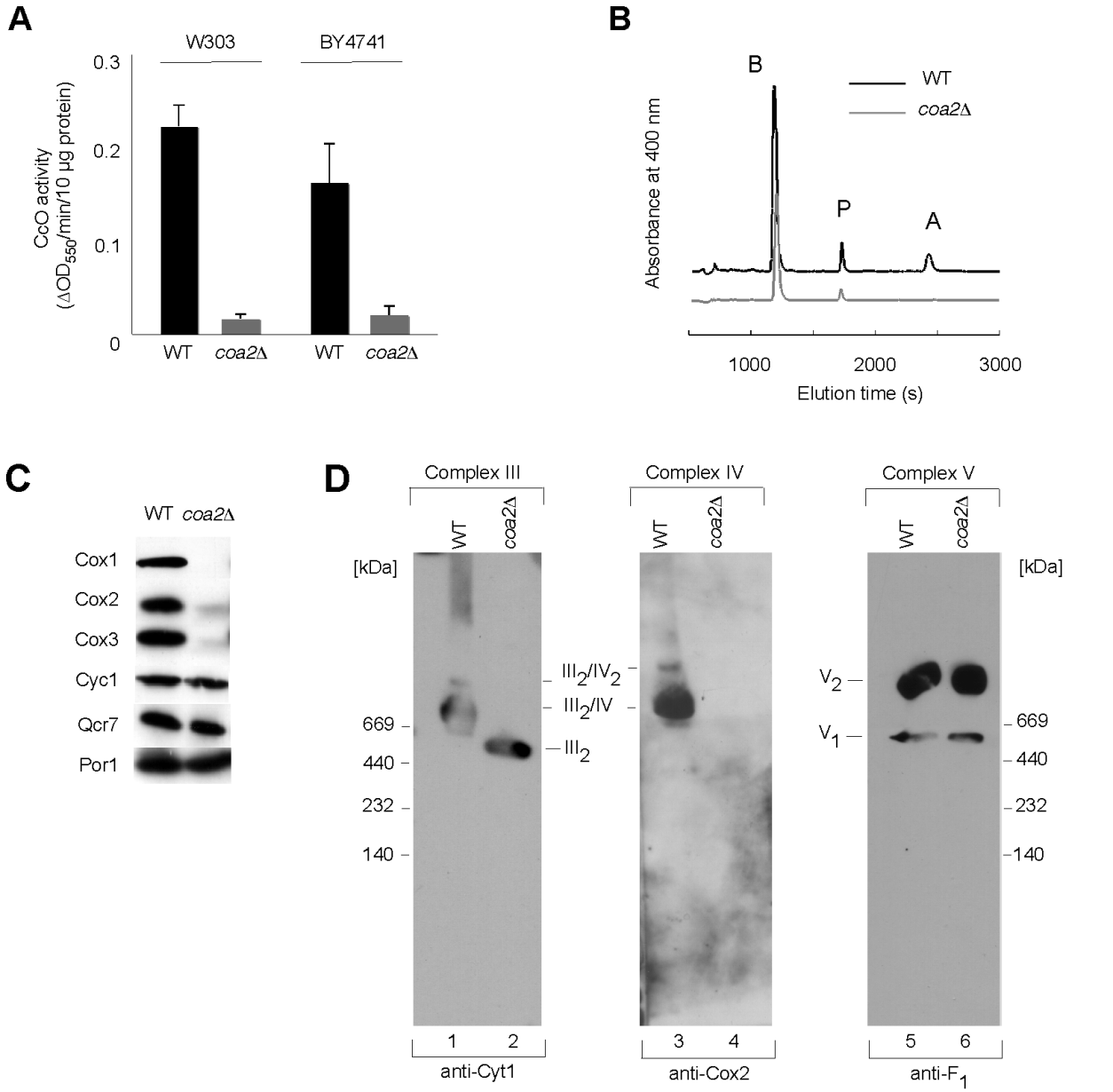

A
W303
BY4741
0.3
0.2
0.1
0
CcO activity
(ΔOD550/min/10 µg protein)
WT
coa2Δ
WT
coa2Δ
B
B
WT
coa2Δ
P
A
Absorbance at 400 nm
1000
2000
3000
Elution time (s)
C
WT coa2Δ
Cox1
Cox2
Cox3
Cyc1
Qcr7
Por1
D
Complex III
Complex IV
Complex V
WT
coa2Δ
[kDa]
669
440
232
140
III2/IV2
III2/IV
III2
V2
V1
1
2
3
4
5
6
anti-Cyt1
anti-Cox2
anti-F1

Figure 3

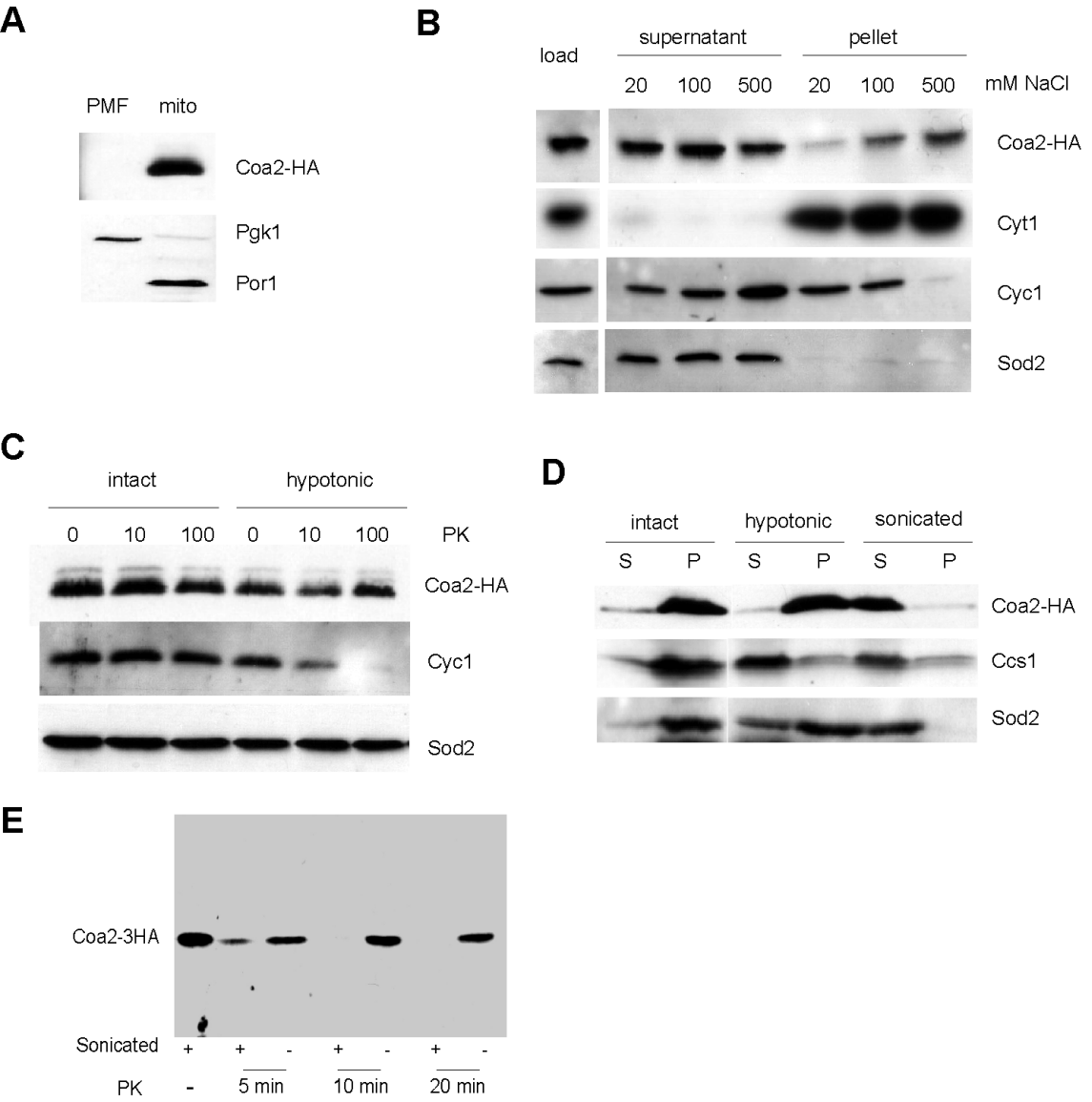

A
PMF
mito
Coa2-HA
Pgk1
Por1
B
load
supernatant
pellet
20
100
500
20
100
500
mM NaCl
Coa2-HA
Cyt1
Cyc1
Sod2
C
intact
hypotonic
0
10
100
0
10
100
PK
Coa2-HA
Cyc1
Sod2
D
intact
hypotonic
sonicated
S
P
S
P
S
P
Coa2-HA
Ccs1
Sod2
E
Coa2-3HA
Sonicated
+
+
-
+
-
+
-
PK
-
5 min
10 min
20 min

Figure 4

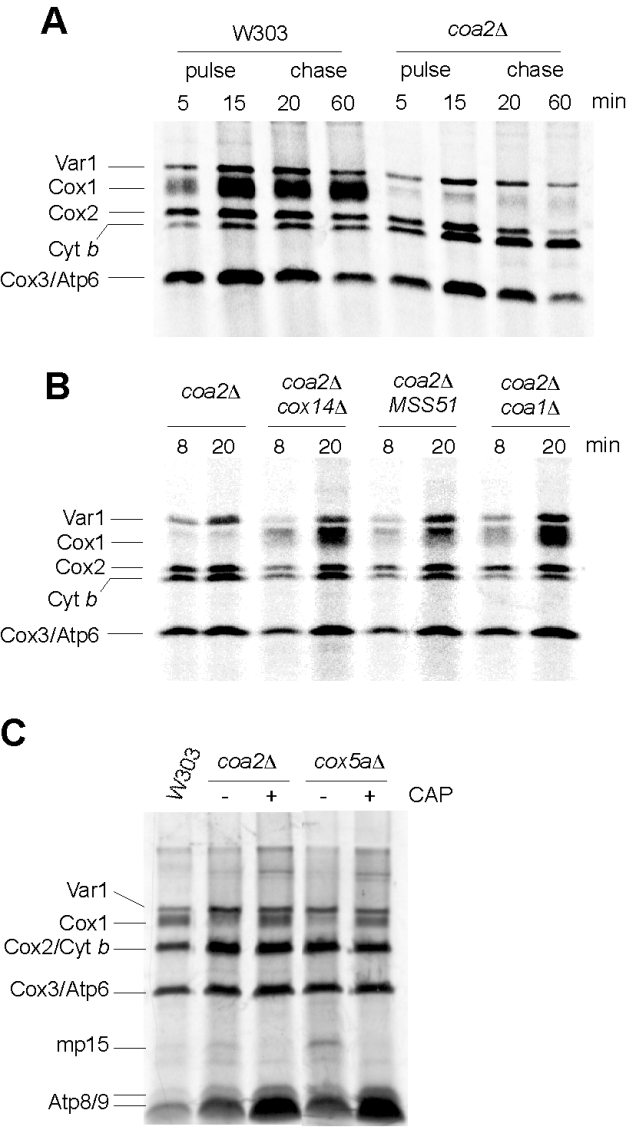

A
W303
coa2Δ
pulse
chase
pulse
chase
5 15 20 60 5 15 20 60 min
Var1
Cox1
Cox2
Cyt b
Cox3/Atp6
B
coa2Δ
coa2Δ cox14Δ
coa2Δ MSS51
coa2Δ coa1Δ
8 20 8 20 8 20 8 20 min
Var1
Cox1
Cox2
Cyt b
Cox3/Atp6
C
W303
coa2Δ
cox5aΔ
- + - + CAP
Var1
Cox1
Cox2/Cyt b
Cox3/Atp6
mp15
Atp8/9

Figure 5

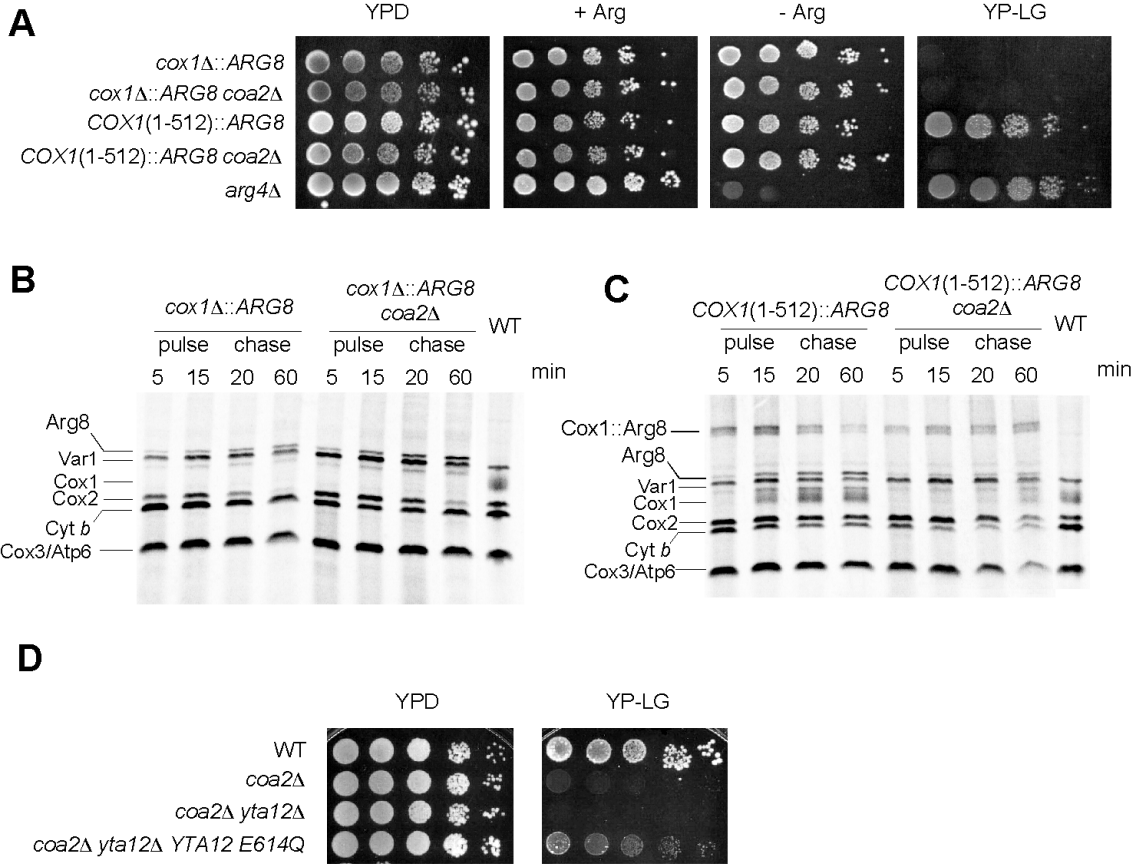

A
YPD
+ Arg
- Arg
YP-LG
cox1Δ::ARG8
cox1Δ::ARG8 coa2Δ
COX1(1-512)::ARG8
COX1(1-512)::ARG8 coa2Δ
arg4Δ
B
cox1Δ::ARG8
cox1Δ::ARG8 coa2Δ
WT
pulse
chase
pulse
chase
5 15 20 60 5 15 20 60 min
Arg8
Var1
Cox1
Cox2
Cyt b
Cox3/Atp6
C
COX1(1-512)::ARG8
COX1(1-512)::ARG8 coa2Δ
WT
pulse
chase
pulse
chase
5 15 20 60 5 15 20 60 min
Cox1::Arg8
Arg8
Var1
Cox1
Cox2
Cyt b
Cox3/Atp6
D
YPD
YP-LG
WT
coa2Δ
coa2Δ yta12Δ
coa2Δ yta12Δ YTA12 E614Q

Figure 6

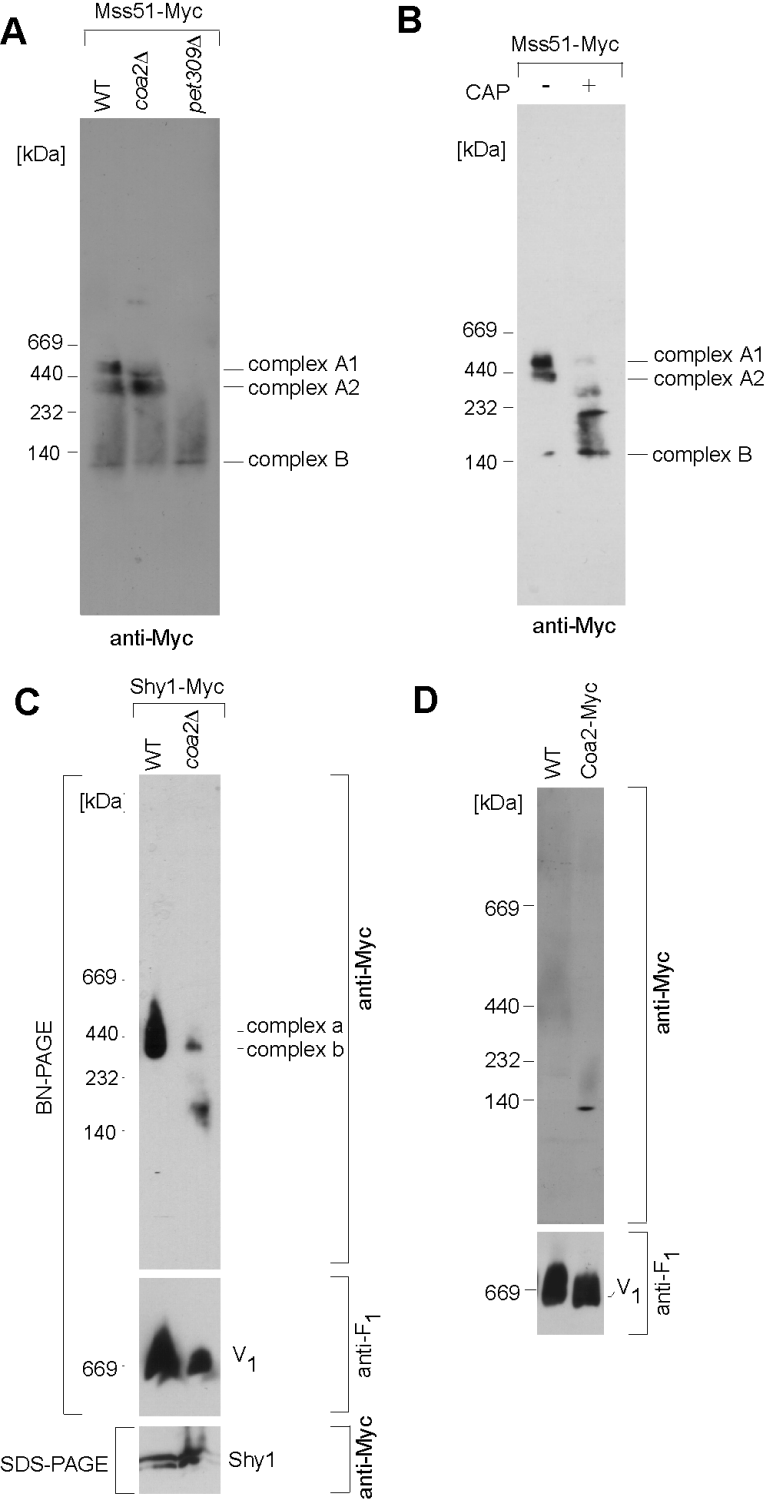

A
Mss51-Myc
WT
coa2Δ
pet309Δ
[kDa]
669
440
232
140
complex A1
complex A2
complex B
anti-Myc
B
Mss51-Myc
CAP
-
+
[kDa]
669
440
232
140
complex A1
complex A2
complex B
anti-Myc
C
Shy1-Myc
WT
coa2Δ
[kDa]
669
440
232
140
BN-PAGE
complex a
complex b
anti-Myc
669
V1
anti-F1
SDS-PAGE
Shy1
anti-Myc
D
WT
Coa2-Myc
[kDa]
669
440
232
140
anti-Myc
669
V1
anti-F1
E
COA2-3HA
+ SHY1-13Myc
SHY1-13Myc
COA2-3HA
IP:HA
Load
Wash
Elution
Load
Wash
Elution
Load
Wash
Elution
anti-Myc
anti-HA

Figure 7

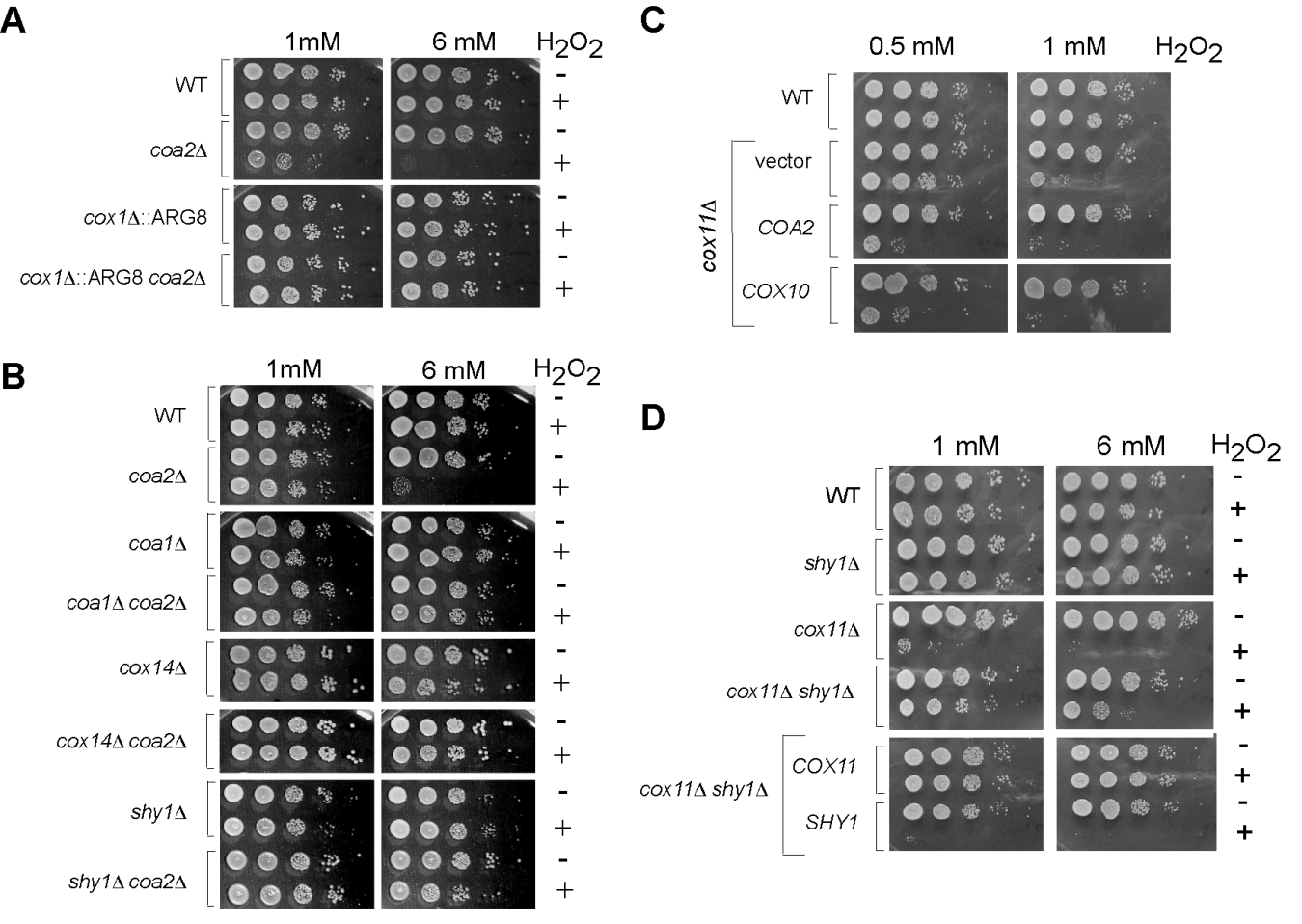

A
1mM
6 mM
H2O2
WT
coa2Δ
cox1Δ::ARG8
cox1Δ::ARG8 coa2Δ
B
1mM
6 mM
H2O2
WT
coa2Δ
coa1Δ
coa1Δ coa2Δ
cox14Δ
cox14Δ coa2Δ
shy1Δ
shy1Δ coa2Δ
C
0.5 mM
1 mM
H2O2
cox11Δ
WT
vector
COA2
COX10
D
1 mM
6 mM
H2O2
WT
shy1Δ
cox11Δ
cox11Δ shy1Δ
cox11Δ shy1Δ
COX11
SHY1

Figure 8

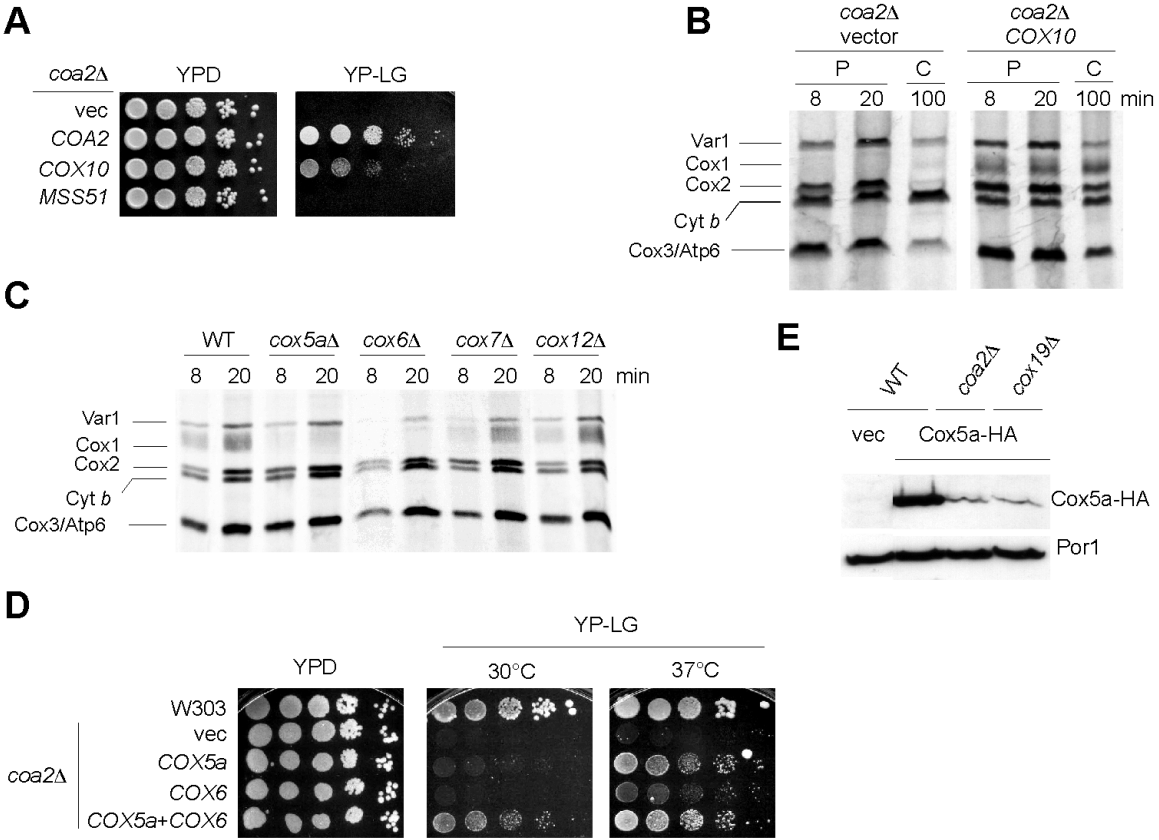

A
coa2Δ
YPD
YP-LG
vec
COA2
COX10
MSS51
B
coa2Δ vector
coa2Δ COX10
P
C
8
20
100
min
Var1
Cox1
Cox2
Cyt b
Cox3/Atp6
C
WT
cox5aΔ
cox6Δ
cox7Δ
cox12Δ
E
WT
coa2Δ
cox19Δ
vec
Cox5a-HA
Por1
D
YP-LG
30°C
37°C
W303
coa2Δ
COX5a
COX6
COX5a+COX6

Figure 9

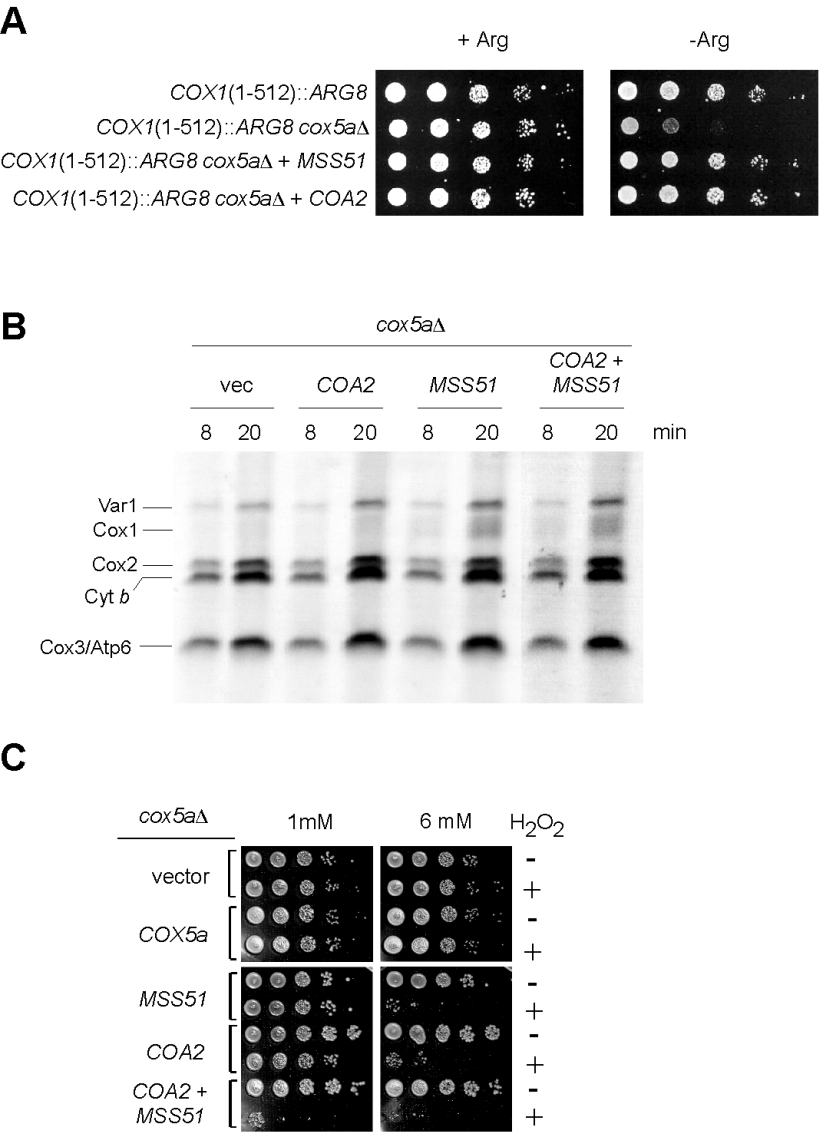

A
+ Arg
-Arg
COX1(1-512)::ARG8
COX1(1-512)::ARG8 cox5aΔ
COX1(1-512)::ARG8 cox5aΔ + MSS51
COX1(1-512)::ARG8 cox5aΔ + COA2
B
cox5aΔ
vec
COA2
MSS51
COA2 + MSS51
8 20 8 20 8 20 8 20 min
Var1
Cox1
Cox2
Cyt b
Cox3/Atp6
C
cox5aΔ
1mM
6 mM
H2O2
vector
COX5a
MSS51
COA2
COA2 + MSS51
- + - + - + - + - +

Figure 10